	Type	Hits	Search Text	DBs
1	BRS	426	myceliophthora near2 thermophila	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
2	BRS	334	S101 and laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
3	BRS	325	S102 and (mutant or variant or modified or modification or mutation or substitution)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
4	BRS	314	S103 and ("93" or "106" or "108" or "109" or "48" or "433" or "500" or "506" or "510" or "514")	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
5	BRS	160	S103 and ("93" or "106" or "108" or "109" or "48" or "433" or "500" or "506" or "510" or "514")	USPAT; EPO
6	BRS	249	S101 near10 laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
7	BRS	121	S106 and ("93" or "106" or "108" or "109" or "48" or "433" or "500" or "506" or "510" or "514")	USPAT; EPO
8	BRS	127	S101 near10 laccase	USPAT; EPO
9	BRS	433	myceliophthora near2 thermophila	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
10	BRS	340	S109 and laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
11	BRS	330	S110 and (mutant or variant or modified or modification or mutation or substitution)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
12	IS&R	3	(("6184015") or ("5985818")).PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
13	IS&R	16	(("6060442") or ("5998353") or ("6184015") or ("6218170") or ("6277611") or ("5972670") or ("5795760") or ("5948121") or ("5981243")).PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
14	BRS	5	"5998353"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB

	Time Stamp
1	2007/07/31 10:07
2	2006/04/25 13:24
3	2006/06/14 12:22
4	2006/04/25 13:24
5	2006/04/25 13:24
6	2006/04/25 13:34
7	2006/04/25 13:24
8	2006/04/25 13:34
9	2006/06/14 12:22
10	2006/06/14 12:22
11	2006/06/14 12:43
12	2006/06/14 12:36
13	2006/06/14 12:49
14	2006/06/14 12:46

	Type	Hits	Search Text	DBs
15	BRS	3	"5925554"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
16	BRS	48	laccase near10 detergent	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
17	BRS	222	S118 and (variant or mutant or modified or mutation or modification)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
18	BRS	242	laccase near20 detergent	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
19	BRS	932	laccase and detergent	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
20	BRS	2768	laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
21	BRS	5	"5998353"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
22	BRS	386	S123 and (mutant or variant or modified or modification or mutation)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
23	BRS	446	S120 and thermophila	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
24	BRS	402	S122 and myceliophthora	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
25	BRS	360	S124 and (myceliophthora near2 thermophila)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
26	BRS	8	"5985818"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
27	IS&R	20	(("5925554") or ("5985818") or ("5998353") or ("6060442") or ("6559859") or ("6184015") or ("6218170") or ("6277611") or ("5770419") or ("5972670") or ("5795760")).PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
28	BRS	4	"7,056,714"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
29	BRS	3	"7,109,010"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB

	Time Stamp
15	2006/06/14 12:46
16	2006/06/14 13:58
17	2006/06/14 13:58
18	2006/06/14 13:58
19	2006/06/14 13:57
20	2006/12/11 14:30
21	2006/12/11 14:38
22	2006/12/11 14:40
23	2006/12/11 14:39
24	2006/12/11 14:39
25	2006/12/11 14:41
26	2006/12/11 14:45
27	2006/12/18 08:46
28	2006/12/11 15:27
29	2006/12/11 15:27

	Type	Hits	Search Text	DBs
30	BRS	3	"20030074698"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
31	BRS	3	"20050044594"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
32	BRS	3	"6140092"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
33	BRS	10	"6218170" or "6060442"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
34	IS&R	5	(("5925554") or ("5998353") or ("6559859") or ("6184015") or ("5972670")).PN.	USPAT; USOCR
35	IS&R	1	("6140092").PN.	USPAT; USOCR
36	IS&R	5	(("5925554") or ("5998353") or ("6559859") or ("6184015") or ("5972670")).PN.	USPAT; USOCR
37	BRS	2	11 and surfactant	USPAT
38	BRS	3	11 and detergent	USPAT
39	BRS	2	14 and (cleaning or surfactant or detergent)	USPAT
40	BRS	2	"6140092"	USPAT

	Time Stamp
30	2006/12/12 14:27
31	2006/12/12 14:27
32	2006/12/18 08:46
33	2007/07/24 14:16
34	2007/07/31 12:48
35	2007/07/31 10:10
36	2007/07/31 12:48
37	2007/07/31 12:49
38	2007/07/31 12:58
39	2007/07/31 12:59
40	2007/07/31 13:08

RESULT 3
LAC2_PODAN

```
ID   LAC2_PODAN     STANDARD;      PRT;   621 AA.
AC   P78722;
DT   01-NOV-1997, integrated into UniProtKB/Swiss-Prot.
DT   01-MAY-1997, sequence version 1.
DT   07-FEB-2006, entry version 42.
DE   Laccase II precursor (EC 1.10.3.2) (Benzenediol:oxygen oxidoreductase)
DE   (Urishiol oxidase) (Diphenol oxidase) (Laccase C).
GN   Name=LAC2;
OS   Podospora anserina.
OC   Eukaryota; Fungi; Ascomycota; Pezizomycotina; Sordariomycetes;
OC   Sordariomycetidae; Sordariales; Lasiosphaeriaceae; Podospora.
OX   NCBI_TaxID=5145;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA].
RC   STRAIN=ATCC 26003;
RX   MEDLINE=97071669; PubMed=8914515; DOI=10.1007/s004380050261;
RA   Fernandez-Larrea J., Stahl U.;
RT   "Isolation and characterization of a laccase gene from Podospora
RT   anserina.";
RL   Mol. Gen. Genet. 252:539-551(1996).
CC   -!- FUNCTION: Probably involved in lignin degradation and in the
CC       detoxification of lignin-derived products in its natural habitat
CC       (herbivorous dung), which is rich in lignin of grasses and straw.
CC       Probably involved in melanin synthesis and in perithecia
CC       development.
CC   -!- CATALYTIC ACTIVITY: 4 benzenediol + O(2) = 4 benzosemiquinone + 2
CC       H(2)O.
CC   -!- COFACTOR: Binds 4 copper ions per monomer (By similarity).
CC   -!- SUBUNIT: Monomer.
CC   -!- SUBCELLULAR LOCATION: Secreted protein.
CC   -!- DEVELOPMENTAL STAGE: Low basic levels throughout the growth phase;
CC       increases at least 20-fold at the beginning of the autolytic phase
CC       and decreases again thereafter.
CC   -!- INDUCTION: Under oxidative stress on the mycelium by aromatic
CC       xenobiotics (guaiacol, hydroquinone, benzoquinone), and by copper
CC       salt at a concentration of 1mM (growing mycelium).
CC   -!- PTM: Proteolytically processed at both its N-terminus and its C-
CC       terminus.
CC   -!- MISCELLANEOUS: Podospora anserina contains at least 3 laccase
CC       isozymes named I, II, and III. They differ in their substrate
CC       specificity, number of subunits, isoelectronic point and heat
CC       stability.
CC   -!- SIMILARITY: Belongs to the multicopper oxidase family.
```

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CC   -!- SIMILARITY: Contains 3 plastocyanin-like domains.
CC   -----------------------------------------------------------------------
CC   Copyrighted by the UniProt Consortium, see http://www.uniprot.org/terms
CC   Distributed under the Creative Commons Attribution-NoDerivs License
CC   -----------------------------------------------------------------------
DR   EMBL; Y08827; CAA70061.1; -; Genomic_DNA.
DR   PIR; S72493; S72493.
DR   HSSP; Q9Y780; 1HFU.
DR   SMR; P78722; 46-605.
DR   InterPro; IPR001117; Cu-oxidase.
DR   InterPro; IPR011706; Cu-oxidase_2.
DR   InterPro; IPR011707; Cu-oxidase_3.
DR   InterPro; IPR002355; Cu_oxidase_Cu_BS.
DR   Pfam; PF00394; Cu-oxidase; 1.
DR   Pfam; PF07731; Cu-oxidase_2; 1.
DR   Pfam; PF07732; Cu-oxidase_3; 1.
DR   PROSITE; PS00079; MULTICOPPER_OXIDASE1; 1.
DR   PROSITE; PS00080; MULTICOPPER_OXIDASE2; 1.
KW   Copper; Glycoprotein; Lignin degradation; Melanin biosynthesis;
KW   Metal-binding; Oxidoreductase; Repeat; Signal.
FT   SIGNAL         1     23       Potential.
FT   PROPEP        24     48       Potential.
FT                                 /FTId=PRO_0000002931.
FT   CHAIN         49    605       Laccase II.
FT                                 /FTId=PRO_0000002932.
FT   PROPEP       606    621       Potential.
FT                                 /FTId=PRO_0000002933.
FT   DOMAIN        78    201       Plastocyanin-like 1.
FT   DOMAIN       210    367       Plastocyanin-like 2.
FT   DOMAIN       430    566       Plastocyanin-like 3.
FT   METAL        138    138       Copper 1 (type 2) (By similarity).
FT   METAL        140    140       Copper 2 (type 3) (By similarity).
FT   METAL        183    183       Copper 2 (type 3) (By similarity).
FT   METAL        185    185       Copper 3 (type 3) (By similarity).
FT   METAL        476    476       Copper 4 (type 1) (By similarity).
FT   METAL        479    479       Copper 1 (type 2) (By similarity).
FT   METAL        481    481       Copper 3 (type 3) (By similarity).
FT   METAL        548    548       Copper 3 (type 3) (By similarity).
FT   METAL        549    549       Copper 4 (type 1) (By similarity).
FT   METAL        550    550       Copper 2 (type 3) (By similarity).
FT   METAL        554    554       Copper 4 (type 1) (By similarity).
FT   CARBOHYD     133    133       N-linked (GlcNAc. . .) (Potential).
FT   CARBOHYD     261    261       N-linked (GlcNAc. . .) (Potential).
FT   CARBOHYD     276    276       N-linked (GlcNAc. . .) (Potential).
FT   CARBOHYD     289    289       N-linked (GlcNAc. . .) (Potential).
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FT   CARBOHYD    325    325        N-linked (GlcNAc. . .) (Potential).
FT   CARBOHYD    334    334        N-linked (GlcNAc. . .) (Potential).
FT   CARBOHYD    401    401        N-linked (GlcNAc. . .) (Potential).
FT   CARBOHYD    421    421        N-linked (GlcNAc. . .) (Potential).
FT   CARBOHYD    441    441        N-linked (GlcNAc. . .) (Potential).
SQ   SEQUENCE   621 AA;  68129 MW;  79F85E2ED25C1CA7 CRC64;

  Query Match             70.7%;  Score 2231.5;  DB 1;  Length 621;
  Best Local Similarity   68.9%;  Pred. No. 5.6e-150;
  Matches  397;  Conservative   65;  Mismatches  109;  Indels    5;  Gaps    4;

Qy          1 QQSCNTPSNRACWTDGYDINTDYEVDSPDTGVVRPYTLTLTEVDNWTGPDGVVKEKVMLV 60
                | ||:| :|||||  |:||||||||  :|:||| | |||||||||||| ||||||||:|||||
Db         46 QSSCHTAANRACWAPGFDINTDYEVSTPNTGVTRTYTLTLTEVDNWLGPDGVVKQKVMLV 105

Qy         61 NNSIIGPTIFADWGDTIQVTVINNLETNGTSIHWHGLHQKGTNLHDGANGITECPIPPKG 120
              |  | |||| |:||| ||| ||||| |||||||||||||||||:||||||:|||||||||
Db        106 NGDIFGPTITANWGDWIQVNVINNLRTNGTSIHWHGLHQKGTNMHDGANGVTECPIPPKG 165

Qy        121 GRKVYRFKAQQYGTSWYHSHFSAQYGNGVVGAIQINGPASLPYDTDLGVFPISDYYYSSA 180
              | ::|||:||||||||||||||||||||||| | :|||||:||| |||||||:|||:   |
Db        166 GSRIYRFRAQQYGTSWYHSHFSAQYGNGVVGTIVVNGPASVPYDIDLGVFPITDYYHKPA 225

Qy        181 DELVELTKNSGAPFSDNVLFNGTAKHPETGEGEYANVTLTPGRRHRLRLINTSVENHFQV 240
              | ||| | | | | || ||| |   |:|:|| |::||||||||:|||||:||||   :|||:
Db        226 DVLVEETMNGGPPPSDTVLFKGHGKNPQTGAGKFANVTLTPGKRHRLRIINTSTHDHFQL 285

Qy        241 SLVNHTMCIIAADMVPVNAMTVDSLFLGVGQRYDVVIEANRTPGNYWFNVTFGGGLLCGG 300
                | |||| ||||||||| | ||||||| ||||||| |:||:: |||||| |||||| ||
Db        286 KLQNHTMTIIAADMVPVQAQTVDSLFLAVGQRYDVTIDANKSVGNYWFNATFGGGLACGA 345

Qy        301 SRNPYPAAIFHYAGAPGGPPTDEGKAPVDHNCLDLPNLKPVVARDVPLSGFAKRADNTLD 360
              | ||:|||:| | |||     ||: |     | ||:|| || |||:| || |||  | :|||
Db        346 SLNPHPAAVFRYQGAPNTLPTNIGTPAADANCMDLNNLTPVVSRSVPTSGFTPRPNNTLP 405

Qy        361 VTLDTTGTPLFVWKVNGSAINIDWGRAVVDYVLTQNTSFPPGYNIVEVNGADQWSYWLIE 420
              |:|   ||||||||||||:||:|| : :||||: ||||:||  |:: ||  :||:|||||
Db        406 VSLTLGGTPLFVWKVNGSSINVDWDKPIVDYVIAQNTSYPPQANVITVNSVNQWTYWLIE 465

Qy        421 NDPGAPFTLPHPMHLHGHDFYVLGRSPDESPAS--NERHVFDPARDAGLLSGANPVRRDV 478
              |||  |||::|||||||||||| |:|||||: ||      |: |:|| |  ||  :|||||||
Db        466 NDPTGPFSIPHPMHLHGHDFLVVGRSPDQ-PAGVPQTRYRFNPATDMALLKSSNPVRRDV 524

Qy        479 SMLPAFGWVVLSFRADNPGAWLFHCHIAWHVSGGLGVVYLERADDLRGAVSDADADDLDR 538
              :|||| ||::::|::||||||||||||||||||| | |||| :|||     | || :  :
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Db         525 AMLPANGWLLIAFKSDNPGAWLFHCHIAWHVSGGLSVQYLERPNDLRNGFSQADKNQHNN 584

Qy         539 LCADWRRYWPTNPYPKSDSGLK-HRWV-EEGEWLVK 572
                |  || ||||||:|| |||||  :|| |  :| :|
Db         585 NCNAWRAYWPTNPFPKIDSGLKVKKWVGEHPDWYIK 620
```

RESULT 2
KSNCLO
laccase (EC 1.10.3.2) precursor - Neurospora crassa (strain OR)
N;Alternate names: urishiol oxidase
C;Species: Neurospora crassa
C;Date: 30-Sep-1991 #sequence_revision 30-Sep-1991 #text_change 09-Jul-2004
C;Accession: A28523; A29762
R;Germann, U.A.; Mueller, G.; Hunziker, P.E.; Lerch, K.
J. Biol. Chem. 263, 885-896, 1988
A;Title: Characterization of two allelic forms of Neurospora crassa laccase. Amino- and carboxyl-terminal processing of a precursor.
A;Reference number: A28523; MUID:88087214; PMID:2961749
A;Accession: A28523
A;Molecule type: DNA
A;Residues: 1-619 <GER>
A;Cross-references: UNIPROT:P06811; EMBL:M14554
R;Germann, U.A.; Lerch, K.
Proc. Natl. Acad. Sci. U.S.A. 83, 8854-8858, 1986
A;Title: Isolation and partial nucleotide sequence of the laccase gene from Neurospora crassa: amino acid sequence homology of the protein to human ceruloplasmin.
A;Reference number: A29762; MUID:87067412; PMID:2947240
A;Accession: A29762
A;Molecule type: DNA
A;Residues: 379-619 <GE2>
A;Cross-references: GB:M14554; NID:g168823; PIDN:AAA33590.1; PID:g168824
C;Comment: This enzyme, which catalyzes the oxidation of benzendiol to benzosemiquinone by molecular oxygen, is a multicopper oxidase containing type 1, type 2, and type 3 copper centers.
C;Genetics:
A;Introns: 86/3
C;Superfamily: laccase
C;Keywords: copper; glycoprotein; oxidoreductase
F;1-21/Domain: signal sequence #status predicted <SIG>
F;22-49/Domain: propeptide #status predicted <PRO>
F;50-619/Product: laccase #status predicted <MAT>
F;84-215/Domain: amino-terminal beta-barrel #status predicted <BB1>
F;216-372/Domain: middle beta-barrel #status predicted <BB2>
F;431-580/Domain: carboxyl-terminal beta-barrel #status predicted <BB3>
F;139,282,295,340,422,444/Binding site: carbohydrate (Asn) (covalent) #status predicted
F;144,480/Binding site: copper (His) (type 2) #status predicted
F;146,189,191,482,548,550/Binding site: 2Cu-O cluster (His) (copper type 3) #status predicted

```
F;477,549,554/Binding site: copper (His, Cys, His) (type 1) #status predicted

  Query Match             64.3%;  Score 2032;  DB 1;  Length 619;
  Best Local Similarity   64.3%;  Pred. No. 1.1e-140;
  Matches  359;  Conservative   80;  Mismatches  113;  Indels    6;  Gaps    2;

Qy      4 CNTPSNRACWTDGYDINTDYEVDSPDTGVVRPYTLTLTEVDNWTGPDGVVKEKVMLVNNS 63
          ||:|:|| ||: |::||||||: :|:||  | | ||||| ||| |||||:|:|||:||:
Db     55 CNSPTNRQCWSPGFNINTDYELGTPNTGKTRRYKLTLTETDNWIGPDGVIKDKVMMVNDK 114

Qy     64 IIGPTIFADWGDTIQVTVINNLETNGTSIHWHGLHQKGTNLHDGANGITECPIPPKGGRK 123
          |||||| ||||| |::|||| |::||||||||||:||: :|: || ||:|||||||:|| |
Db    115 IIGPTIQADWGDYIEITVINKLKSNGTSIHWHGMHQRNSNIQDGVNGVTECPIPPRGGSK 174

Qy    124 VYRFKAQQYGTSWYHSHFSAQYGNGVVGAIQINGPASLPYDTDLGVFPISDYYYSSADEL 183
          |||::| |||||||||||||||||:|| | ||||||  || ||| ||::|||| :|| |
Db    175 VYRWRATQYGTSWYHSHFSAQYGNGIVGPIVINGPASANYDVDLGPFPLTDYYYDTADRL 234

Qy    184 VELTKNSGAPFSDNVLFNGTAKHPETGEGEYANVTLTPGRRHRLRLINTSVENHFQVSLV 243
          | ||:::| | |:|||||| |||| || |:|| |:|| |::||||||||||||||||: ||
Db    235 VLLTQHAGPPPSNNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLLLV 294

Qy    244 NHTMCIIAADMVPVNAMTVDSLFLGVGQRYDVVIEANRTPGNYWFNVTFGGGLLCGGSRN 303
          ||:| ||:||:|||    ||||||||||||||:|:||:  |||||||||||  ||| | |
Db    295 NHSMTIISADLVPVQPYKVDSLFLGVGQRYDVIIDANQAVGNYWFNVTFGGSKLCGDSDN 354

Qy    304 PYPAAIFHYAGAPGGPPTDEGKAPVDHNCLDLPNLKPVVARDVPLSGFAKRADNTLDVTL 363
           |||||| | |||   ||::| ||||| |||| :||||: | :  :  |    ||: :||
Db    355 HYPAAIFRYQGAPKALPTNQGVAPVDHQCLDLNDLKPVLQRSLNTNSIALNTGNTIPITL 414

Qy    364 DTTGTPLFVWKVNGSAINIDWGRAVVDYVLTQNTSFPPGYNIVEVNGADQWSYWLIENDP 423
          |      |||:|||:||||:| : |::|||| ||::    |||:| | :|| ||||||||
Db    415 DG-----FVWRVNGTAININWNKPVLEYVLTGNTNYSQSDNIVQVEGVNQWKYWLIENDP 469

Qy    424 GAPFTLPHPMHLHGHDFYVLGRSPDESPASNERHVFDPARDAGLLSGANPVRRDVSMLPA 483
             |:||||:||||||| :||||| :  |: | ||||| |   |:| || ||| :||||
Db    470 DGAFSLPHPIHLHGHDFLILGRSPDVTAISQTRYVFDPAVDMARLNGNNPTRRDTAMLPA 529

Qy    484 FGWVVLSFRADNPGAWLFHCHIAWHVSGGLGVVYLERADDLRGAVSDADADDLDRLCADW 543
           ||::::|| ||||:|| ||||||||||||   :|||| ||| ::| ||    :  |  |
Db    530 KGWLLIAFRTDNPGSWLMHCHIAWHVSGGLSNQFLERAQDLRNSISPADKKAFNDNCDAW 589

Qy    544 RRYWPTN-PYPKSDSGLK 560
          | |:| | |:|| ||||:
Db    590 RAYFPDNAPFPKDDSGLR 607
```

1449

Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8854–8858, December 1986
Biochemistry

Isolation and partial nucleotide sequence of the laccase gene from *Neurospora crassa*: Amino acid sequence homology of the protein to human ceruloplasmin

(multicopper oxidase/copper binding sites/genomic cloning/protein evolution)

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Communicated by Frank W. Putnam, August 6, 1986

ABSTRACT The laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) gene from *Neurospora crassa* was cloned and part of its nucleotide sequence corresponding to the carboxyl-terminal region of the protein has been determined. The gene was cloned by cDNA synthesis with a laccase-specific synthetic deoxyundecanucleotide as primer and poly(A) RNA isolated from cycloheximide-treated *N. crassa* cultures as template. Based on the nucleotide sequence of the cDNA obtained, a unique 21-mer was synthesized and used to screen a genomic DNA library from *N. crassa*. Five different positive clones were isolated and shown to share an overlapping DNA region with the same pattern of restriction sites. Sequence analysis of the common 1.36-kilobase *Sal* I fragment revealed an open reading frame of 726 nucleotides. The amino acid sequence deduced is in complete agreement with the primary structures of several tryptic peptides isolated previously from *N. crassa* laccase. The analyzed carboxyl-terminal region of laccase exhibits a striking sequence homology to the carboxyl-terminal part of the third homology unit of the multicopper oxidase ceruloplasmin and to a smaller extent, to the low molecular weight blue copper proteins plastocyanin and azurin. Based on amino acid sequence comparison between these proteins, putative copper ligands of *N. crassa* laccase are proposed. Moreover, these data further support the hypothesis that the small blue copper proteins and the multicopper oxidases have evolved from the same ancestral gene.

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belongs to the multicopper oxidases containing three distinct Cu centers known as type 1 or blue, type 2 or normal, and type 3 or coupled binuclear (1). The enzyme catalyzes the four-electron reduction of molecular oxygen to water with the concomitant oxidation of organic substrates. Laccases have been isolated from different sources such as microorganisms (2–8) and plants (9–11). Physical characterization of the proteins indicates a great variety in molecular weight, carbohydrate content, and quaternary structure. With the exception of the laccase from *Agaricus bisporus* and the tetrameric *Podospora anserina* laccase I, all native enzymes bind four copper ions per molecule, representing one type 1, one type 2, and one type 3 Cu center (12). In contrast, the other multicopper oxidases, ceruloplasmin and ascorbate oxidase, contain more than four copper ions per molecule (13–17). Hence, laccase provides the most simple system to study the active site structure and reactivity of multicopper oxidases.

Neurospora crassa laccase is excreted into the culture medium after induction with low amounts of protein synthesis inhibitors (18). The enzyme has a molecular mass of 64 kDa, with a carbohydrate content of 11–12% (4, 5). In an attempt to determine the primary structure of *N. crassa* laccase and to study its molecular mechanisms of biosynthesis and excretion, we set out to clone the structural gene. Here we describe the strategy used to clone this gene and present part of its nucleotide sequence. The amino acid sequence deduced is compared to that of other blue copper proteins, such as ceruloplasmin, plastocyanin, and azurin.

MATERIALS AND METHODS

Organism and Conditions of Culture. *N. crassa* wild-type strain FGSC 321 (Fungal Genetics Stock Center, Kansas City, KA) was grown and induced with 2.8 μM cycloheximide as described by Froehner and Eriksson (18).

Protein Chemical Methods. Extracellular *N. crassa* laccase was isolated essentially as described (5) from the culture medium collected 96 hr after induction. Apolaccase was prepared by dialysis of the native enzyme against 5% (vol/vol) formic acid at 4°C for at least 24 hr. The lyophilized metal-free protein was modified by S-carboxymethylation (19). Tryptic digestion of the carboxymethylated apolaccase was performed in 0.1 M ammonium bicarbonate (pH 8.5) with 2% (wt/wt) trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; Worthington) at 37°C. After 1 hr, a second portion of trypsin was added and the mixture was further incubated for 3 hr at 37°C. Peptides were purified by HPLC on a LiChrosorb RP-18 column (0.46 × 25 cm). The buffer system used was buffer A/0.1% (wt/vol) trifluoroacetic acid, pH 1.9, and buffer B [same as A except with 60% (vol/vol) acetonitrile]. Chromatography was carried out using a linear gradient of buffer B (0.5% per min) at 20°C with a flow rate of 1 ml/min. Absorbance was recorded at 220 nm. The peptides obtained were further characterized by amino acid analysis (Durrum Chemical, Palo Alto, CA, D-500 amino acid analyzer) and by automated Edman degradations (Beckman Sequenator model 890-B, updated).

Isolation of Poly(A) RNA. Total cellular RNA was isolated from lyophilized mycelia harvested 48 hr after induction by the method of Lucas *et al.* (20). Poly(A) RNA was selected by oligo(dT)-cellulose chromatography according to Aviv and Leder (21).

Synthesis of Oligodeoxynucleotides and Primer Extension. Oligodeoxynucleotides were synthesized according to the phosphotriester method (22, 23). Dimers were used as starting products, and synthesis was carried out on a manual solid-phase DNA synthesizer (Bachemgentec, Torrance, CA). Oligomers were fully deprotected and subsequently purified by gel filtration on Sephadex G-25 (Pharmacia), followed by gel electrophoresis (20% acrylamide/8 M urea) and chromatography on NACS 52 (Bethesda Research Laboratories).

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Abbreviation: kb, kilobase(s).
*To whom reprint requests should be addressed.

cDNA was synthesized essentially as described by Nathans and Hogness (24) using a sequence-specific oligonucleotide as primer and poly(A) RNA as template. High molecular weight primer extension products were separated by chromatography on Sephacryl S-300 (Pharmacia).

Screening of a Genomic DNA Library. A *N. crassa* genomic DNA library in the plasmid vector pRK9 (25) was provided by M. Schechtman (Syracuse University). This library was used to transform *Escherichia coli* LE 392. Ampicillin-resistant transformants were grown on nitrocellulose filters (Schleicher & Schuell) as described by Hanahan and Meselson (26). Filter replicas were screened by colony hybridization (27), using a 5′-labeled synthetic oligodeoxynucleotide as hybridization probe.

Recombinant Plasmid DNA Isolation and Restriction Endonuclease Digestions. Plasmid DNA was isolated by the method of Birnboim and Doly (28). Restriction enzymes were purchased from Boehringer Mannheim or Anglian Biotechnology (Colchester, UK). Digestions were carried out using assay conditions recommended by the suppliers. The resulting DNA fragments were electrophoresed on agarose and transferred to nitrocellulose (Schleicher & Schuell) (29).

DNA Sequencing. DNA sequence analysis was carried out by either the chemical cleavage (30) or the chain-termination technique (31). Single-stranded templates for the chain-termination method were obtained by molecular cloning of isolated subfragments in the single-stranded bacteriophage vectors M13mp18 or M13mp19 (32). DNA sequences were analyzed with a computer program (33).

RESULTS AND DISCUSSION

Synthesis of Laccase-Specific cDNA by Primer Extension and Screening of a Genomic *Neurospora* DNA Library. Synthetic oligonucleotide primers provide a powerful tool for the efficient detection and transcription of specific mRNA species and thus have been often used to isolate cDNA (e.g., see refs. 34 and 35) or genomic DNA (e.g., see refs. 36 and 37) clones. To design a unique oligonucleotide suitable for priming laccase-specific cDNA synthesis, tryptic peptides were prepared from the carboxymethylated apoprotein. Purified fractions isolated by HPLC were subjected to sequence analysis (data not shown). From the tryptic decapeptide T_5 with the amino acid sequence Ala-Phe-Asn-Asp-Asn-Cys-Asp-Ala-Trp-Arg (Fig. 1*A*), a unique 11-mer (Fig. 1*C*) coding for residues 5–8 within the peptide was deduced. This primer

```
         1   2   3   4   5   6   7   8   9   10
     N- ALA PHE ASN ASP ASN CYS ASP ALA TRP ARG -C    A
     5' GCA UUC AAC GAC AAC UGC GAC GCA UGG AGA 3'    B
          C   U   U   U   U   U   U   C       C C
          G                           G        G
          U                           U        U

                     3' TTG ACG CTG CG 5'             C

5' GAT AAG AAG GCC TTT AAC GAC 3'                     D
N- ASP LYS LYS ALA PHE ASN ASP -C                     E

  3' A TTC TTC CGG AAA TTG CTG TT 5'                  F
```

FIG. 1. Cloning strategy of the *N. crassa* laccase gene. Based on the amino acid sequence of a tryptic decapeptide (*A*) isolated from carboxymethylated apolaccase and the possible mRNA sequences (*B*), a deoxyundecanucleotide (*C*) was synthesized. This 11-mer (*C*) was used to prime the synthesis of cDNA from poly(A) RNA isolated from cycloheximide-treated *N. crassa* cultures. High molecular weight primer extension products were analyzed by nucleotide sequencing (30) (*D*). The deduced amino acid sequence (*E*) was in complete agreement with the partially known primary structure (*A*) of *N. crassa* laccase. This allowed the synthesis of a unique 21-mer (*F*), which was used for the screening of a genomic DNA library of *N. crassa*.

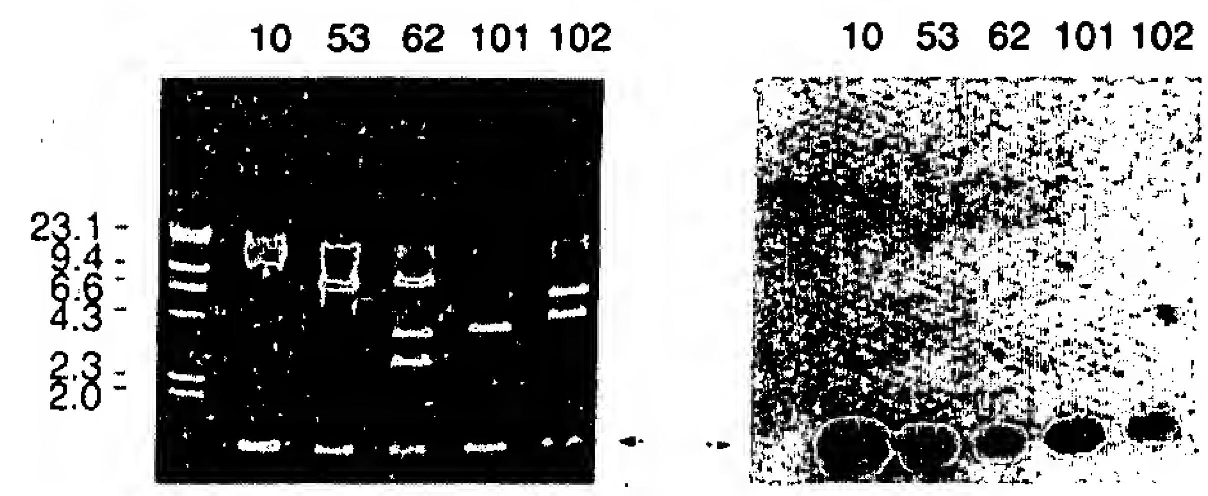

FIG. 2. Digestion of plasmid DNA isolated from five positive clones (nos. 10, 53, 62, 101, and 102) with *Sal* I and hybridization to labeled 21-mer (Fig. 1*F*). (*Left*) Fragments were size-fractionated by gel electrophoresis on 1% agarose and stained with ethidium bromide. (*Right*) DNA was transferred to nitrocellulose (29) and hybridized to ^{32}P-labeled 21-mer. The lengths of size markers (*Hind*-III-digested phage λ DNA) are given in kb.

was selected over the other seven possibilities (Fig. 1*B*) based on the published codon usage in other *N. crassa* nuclear genes (25, 38, 39). Furthermore, possible mismatches were only of the G·U type, building a "wobble" base pair (40), which has been shown to approximate an A·U base pair in stabilizing efficiency (41). Poly(A) RNA isolated from cycloheximide-treated *N. crassa* cultures served as a template for 11-mer-primed cDNA synthesis. The RNA used has earlier been shown to contain laccase mRNA by immunological analysis of *in vitro* translation products.† High molecular weight primer extension products were subjected to sequence analysis by the chemical cleavage technique (30) (Fig. 1*D*). The amino acid sequence deduced from the specifically primed cDNA (Fig. 1*E*) was in complete agreement with the partially known primary structure of *N. crassa* laccase from the priming site toward the amino terminus (Fig. 1*A*). This allowed the synthesis of a unique 21-mer (Fig. 1*F*), which was used as a hybridization probe to screen a partial *Sau*3A genomic DNA library from *N. crassa* (25). From $\approx 6 \times 10^4$ colonies, seven positive clones were isolated. Examination of their plasmid DNA by restriction mapping revealed two pairs of them (nos. 53 and 81, 102 and 113) to be identical. Although the five positive clones nos. 10, 53, 62, 101, and 102 differ in size and the pattern of restriction sites, they were shown to share an overlapping DNA region. *Sal* I restriction endonuclease digests of the five different positive clones are presented in Fig. 2. All clones contain a common 1.36-kilobase (kb) *Sal* I fragment, which strongly hybridizes with the ^{32}P-labeled 21-mer. Hence, this fragment was structurally further characterized.

DNA Sequence Analysis. The 1.36-kb *Sal* I fragment common to all five different positive clones (Fig. 2) was subjected to DNA sequence analysis with the strategy outlined in Fig. 3. Both the chemical cleavage (30) and the chain-termination (31) techniques were used. The 1.36-kb *Sal* I fragment was found to contain an open reading frame of 726 nucleotides starting with the triplet GTA of one of the two *Sal* I restriction sites and ending with the termination signal TAG (Fig. 4), thus encoding the carboxyl-terminal part of a protein. The correct 21-mer (Fig. 1*F*) used to screen the genomic *N. crassa* DNA library could be localized readily. It is interesting to note that the designed 11-mer used for primer extension (Fig. 1*C*) obviously worked with one G·U mismatch. The amino acid sequence deduced from the coding region agrees completely with the primary structures of six previously isolated tryptic peptides (T_1–T_6) of *N. crassa* laccase (Fig. 4). Hence, these results clearly indicate that the deduced amino acid sequence represents part (40–50%) of the

†Germann, U. A., Huber, M. & Lerch, K., 16th Meeting of the Federation of European Biochemical Societies, June 25–30, 1984, Moscow, USSR, p. 278 (abstr.).

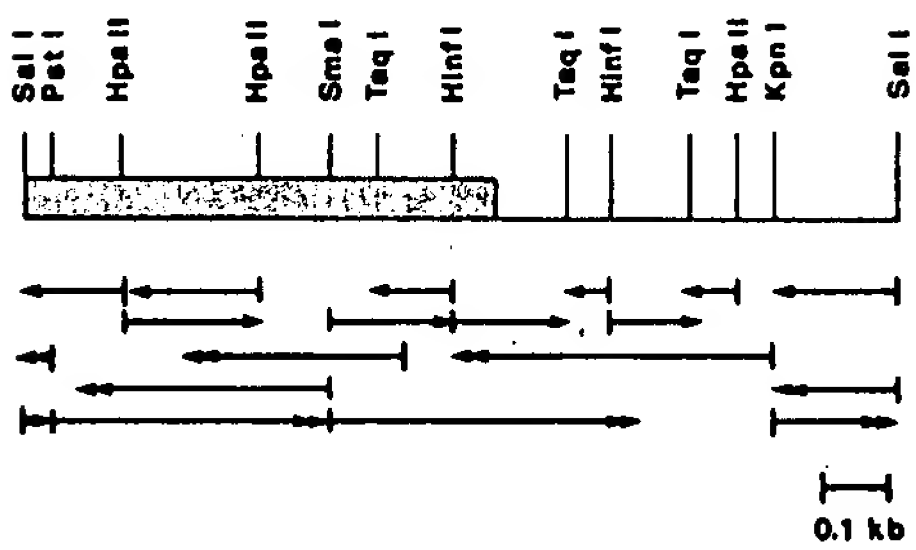

FIG. 3. Strategy of sequence analysis of the 1.36-kb *Sal* I fragment common to the five positive clones (Fig. 2). Only nonfrequent restriction sites and the sites used for sequence analysis are shown. Arrows indicate direction and extent of sequencing. For the chemical cleavage sequencing (30) (→) restriction fragments were labeled at their 5′ ends and were either cut with another restriction enzyme or were subjected to strand separation. Chain-termination sequencing (31) (↠) was performed using the wild-type phages M13mp18 and M13mp19 (32). Shaded bar represents the part coding for the carboxyl-terminal region of *N. crassa* laccase shown in Fig. 4.

primary structure of *N. crassa* laccase. This conclusion is further supported by the data of a preliminary sequence analysis of laccase B from the white rot fungus *Polyporus versicolor* (42). Among three fragments characterized, the active site peptide Leu-His-Cys-His-Ile-Asx-Phe indicates clear homology to the *N. crassa* laccase sequence Met-His-Cys-His-Ile-Ala-Trp. The alignment of the two peptides reveals four amino acid residues to be identical and two to be isofunctional (Leu and Met, Phe and Trp).

Sequence Comparison of *N. crassa* Laccase with Other Blue Copper Proteins. In Fig. 5, part of the amino acid sequence of *N. crassa* laccase is compared with homologous parts of human ceruloplasmin (43) and with the primary structures of plastocyanin from *Populus nigra* var. *italica* (44) and azurin from *Pseudomonas aeruginosa* (45). Ceruloplasmin is so far the only multicopper oxidase with a completely known primary structure (43). The single polypeptide chain consists of 1046 amino acid residues and exhibits a 3-fold internal homology in amino acid sequence (46, 51). Six (or seven) copper ions are bound per molecule—namely, two type 1, one type 2, and three (or four) type 3 (13–15). Plastocyanin and azurin are low molecular weight blue copper proteins binding only one type 1 copper ion per molecule (52, 53). Amino acid sequences of azurin and plastocyanin from a wide variety of sources have been determined (52, 53). Both poplar plastocyanin and azurin from *P. aeruginosa* have been crystallized and their three-dimensional structures as well as their copper ligands are known (49, 50). Plastocyanin and azurin have been shown to be divergently related (54), and in several reports it has been suggested that the small blue copper proteins and the multicopper oxidases share a common ancestry (47, 55, 56). The amino acid sequence comparison shown in Fig. 5 further supports this hypothesis. The primary structure of *N. crassa* laccase is highly homologous to the carboxyl-terminal part of ceruloplasmin (CpC) and to a smaller extent to the other two homology units (CpN, CpM) of ceruloplasmin and to plastocyanin and azurin. In all six polypeptides compared, five amino acid residues are invariant (one Cys, two Gly, two His) and four are isofunctional having a conserved aromatic (three Phe, Trp, or Tyr) or hydrophobic (one Leu or Met) character. Among the invariant ones, three represent ligands of the type 1 copper in plastocyanin (His-37, Cys-84, and His-87) and azurin (His-46, Cys-112, and His-117). The fourth blue copper ligand in plastocyanin and azurin is Met-92 and Met-121, respectively. The corresponding residues Met-690 and Met-1031 are found only in CpM and CpC and represent the putative fourth binding sites for the two type 1 copper ions of human ceruloplasmin (51). Surprisingly, however, *N. crassa* laccase lacks a methionine residue at the homologous position. Hence, this finding implicates a type 1 copper coordination different from plastocyanin and azurin, and possibly from ceruloplasmin. In this context, it is interesting to note that stellacyanin, a small blue copper protein isolated from *Rhus vernicifera* (57) is also devoid of methionine. The nature of its fourth copper ligand is presently still a matter of debate (58).

As mentioned above, the highest overall sequence homology is found when *N. crassa* laccase is compared with the carboxyl-terminal region of human ceruloplasmin. Thus, 114 amino acid residues of the third homology unit (CpC) of ceruloplasmin can be aligned with part of the primary structure of laccase to give 27 identical and 12 isofunctional amino acid residues (Fig. 5). Most interesting, two short

```
                                    30                                      60                                      90
GTC GAC CAC CAA TGC CTG GAC CTC AAC GAC CTG AAG CCC GTC CTG CAG CGC AGC CTG AAC ACC AAC AGC ATC GCA CTC AAC ACT GGC AAC ACG
                                    10                                      20                                      30
Val Asp His Gln Cys Leu Asp Leu Asn Asp Leu Lys Pro Val Leu Gln Arg Ser Leu Asn Thr Asn Ser Ile Ala Leu Asn Thr Gly Asn Thr

                                120                                     150                                     180
ATC CCC ATC ACG CTC GAC GGG TTC GTC TGG CGG GTC AAT GGA ACG GCC ATC AAC ATC AAC TGG AAC AAG CCG GTG CTG GAG TAC GTG CTG ACG
                                40                                      50                                      60
Ile Pro Ile Thr Leu Asp Gly Phe Val Trp Arg Val Asn Gly Thr Ala Ile Asn Ile Asn Trp Asn Lys Pro Val Leu Glu Tyr Val Leu Thr

                            210                                     240                                     270
GGC AAC ACC AAC TAC TCG CAG TCC GAC AAC ATT GTG CAG GTC GAG GGC GTC AAC CAA TGG AAG TAC TGG CTC ATC GAG AAC GAC CCC GAC GGT
                            70                                      80                                      90
Gly Asn Thr Asn Tyr Ser Gln Ser Asp Asn Ile Val Gln Val Glu Gly Val Asn Gln Trp Lys Tyr Trp Leu Ile Glu Asn Asp Pro Asp Gly

                        300                                     330                                     360
GCC TTT AGC CTG CCG CAT CCC ATC CAC CTG CAC GGC CAC GAC TTT CTC ATC CTC GGT CGG TCG CCC GAC GTG ACC GCC ATC AGC CAG ACG CGC
                        100                                     110                                     120
Ala Phe Ser Leu Pro His Pro Ile His Leu His Gly His Asp Phe Leu Ile Leu Gly Arg Ser Pro Asp Val Thr Ala Ile Ser Gln Thr Arg
                                                                                                    T1
                    390                                     420                                     450
TAC GTG TTT GAC CCG GCC GTG GAC ATG GCT CGG TTG AAC GGC AAC AAC CCG ACG CGG CGC GAC ACG GCC ATG CTC CCG GCC AAG GGC TGG CTG
                    130                                     140                                     150
Tyr Val Phe Asp Pro Ala Val Asp Met Ala Arg Leu Asn Gly Asn Asn Pro Thr Arg Arg Asp Thr Ala Met Leu Pro Ala Lys Gly Trp Leu
                                                                                            T2
                480                                     510                                     540
CTC ATC GCG TTC CGC ACC GAC AAT CCC GGG TCG TGG CTG ATG CAC TGC CAC ATT GCC TGG CAC GTG TCG GGA GGT CTG TCG AAC CAG TTC CTG
                160                                     170                                     180
Leu Ile Ala Phe Arg Thr Asp Asn Pro Gly Ser Trp Leu Met His Cys His Ile Ala Trp His Val Ser Gly Gly Leu Ser Asn Gln Phe Leu

            570                                     600                                     630
GAG CGG GCG CAG GAC CTC AGA AAC AGT ATC AGC CCT GCG GAT AAG AAG GCC TTT AAC GAC AAC TGT GAC GCG TGG AGG GCG TAC TTC CCT GAC
            190                                     200                                     210
Glu Arg Ala Gln Asp Leu Arg Asn Ser Ile Ser Pro Ala Asp Lys Lys Ala Phe Asn Asp Asn Cys Asp Ala Trp Arg Ala Tyr Phe Pro Asp
                T3                          T4                                      T5                                  T6
        660                                     690                                     720
AAT GCG CCG TTC CCC AAG GAC GAC TCT GGC CTG AGG AGC GGT GTC AAG GCG AGG GAG GTG AAG ATG AAG TGG TAG
        220                                     230                                     240
Asn Ala Pro Phe Pro Lys Asp Asp Ser Gly Leu Arg Ser Gly Val Lys Ala Arg Glu Val Lys Met Lys Trp ***
T6
```

FIG. 4. Primary structure of the coding part found within the 1.36-kb *Sal* I fragment (Fig. 3) and deduced amino acid sequence. The nucleotides complementary to the 21-mer (Fig. 1F), which was used to screen the genomic *N. crassa* DNA library, are boxed. Shaded bars (T_1–T_6) indicate tryptic peptides that were isolated previously from carboxymethylated apolaccase and characterized by amino acid and sequence analysis.

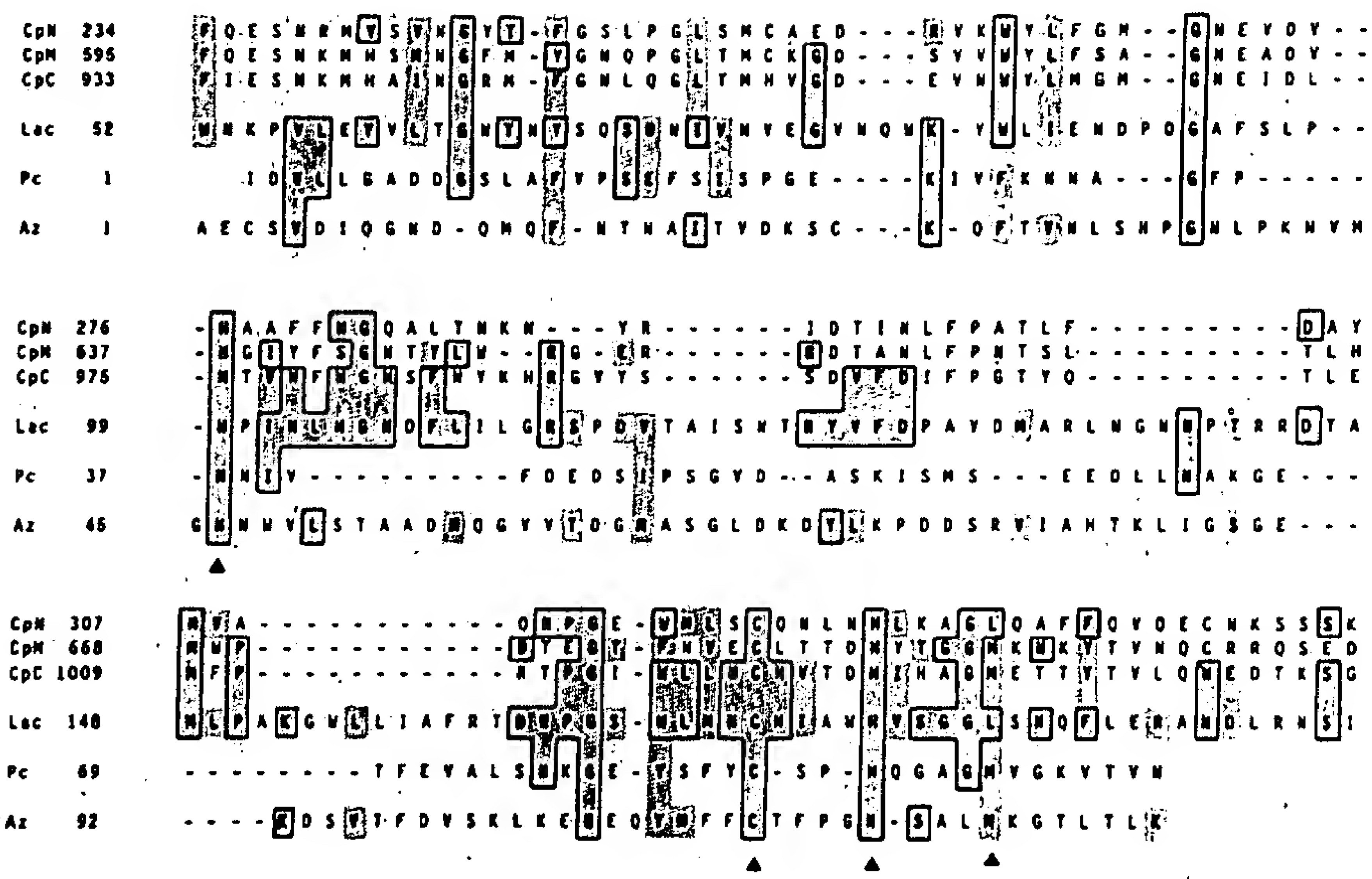

FIG. 5. Amino acid sequence comparison between part of *N. crassa* laccase (Lac) and homologous parts of other blue copper proteins. Cp, human ceruloplasmin (43); CpN, first homology unit residues 234–344; CpM, second homology unit residues 595–705; CpC, third homology unit residues 933–1046; Pc, poplar plastocyanin (44); Az, *P. aeruginosa* azurin (45). The three homology units CpN, CpM, and CpC of ceruloplasmin were aligned as described by Takahashi *et al.* (46). CpC and Pc were aligned according to Rydén (47) and Pc and Az were aligned according to Murata *et al.* (48) with some modifications maximizing the sequence homology of each primary structure to that of *N. crassa* laccase. The positions of the four copper ligands in Pc (49) and Az (50) are marked by triangles. The number on the left of each sequence identifies the position within the protein of the first amino acid residue shown. For Lac, the numbers refer to valine as 1 according to Fig. 4. Amino acid residues are abbreviated using the one-letter code. Dashes represent gaps introduced for alignments. Boxes relate amino acid residues of Lac with identical residues in CpN, CpM, CpC, Pc, and/or Az. Shaded areas, isofunctional residues (D and E; K and R; T and S; F, W, and Y; I, L, M, and V).

stretches show extensive sequence homology. A computer analysis using the unitary matrix indicated maximal homology scores of 6 and 9 for the segments presented in Fig. 6 *A* and *B*, respectively (N. Takahashi and F. W. Putnam, personal communication). The remarkable similarity of these two regions suggests that they play an important role in the formation of the active site of multicopper oxidases. In particular, they could provide ligands to all three different types of copper in both enzymes. This suggestion is consistent with the observation of Raju (59) that 50% of the non-blue copper of the ceruloplasmin molecule resides in the carboxyl-terminal tryptic peptide of 11 kDa. As pointed out above, at least three invariant type 1 copper binding residues are present within these two stretches. The fourth type 1 copper ligand in *N. crassa* laccase is currently not known (see above). It is conceivable, however, that the nearby Met-169 of *N. crassa* laccase might be involved in copper binding. The idea that also type 2 or type 3 copper ligands could be provided by the two homologous regions shown in Fig. 6 is based on earlier reports. The sequence containing the histidine cluster of ceruloplasmin (Fig. 6*A*) has been shown to be homologous to that providing ligands to the non-blue copper site in Cu/Zn-superoxide dismutase (51). Furthermore, using the coordinates of the known three-dimensional structure of poplar plastocyanin (49), Rydén recently presented a three-dimensional model of the copper active site of human ceruloplasmin (47). The model involves the carboxyl-terminal region (amino acid residues 932–1040; ref. 43) and proposes His-975, Cys-1021, His-1026, and Met-1031 as type 1; His-1022 and His-1028 as type 2; and His-978, His-980, His-982, and His-1020 as type 3 copper ligands. The amino acid sequence comparison of ceruloplasmin with laccase supports this model with respect to the type 1 and the type 3 copper ligands. However, no histidine residue is found in

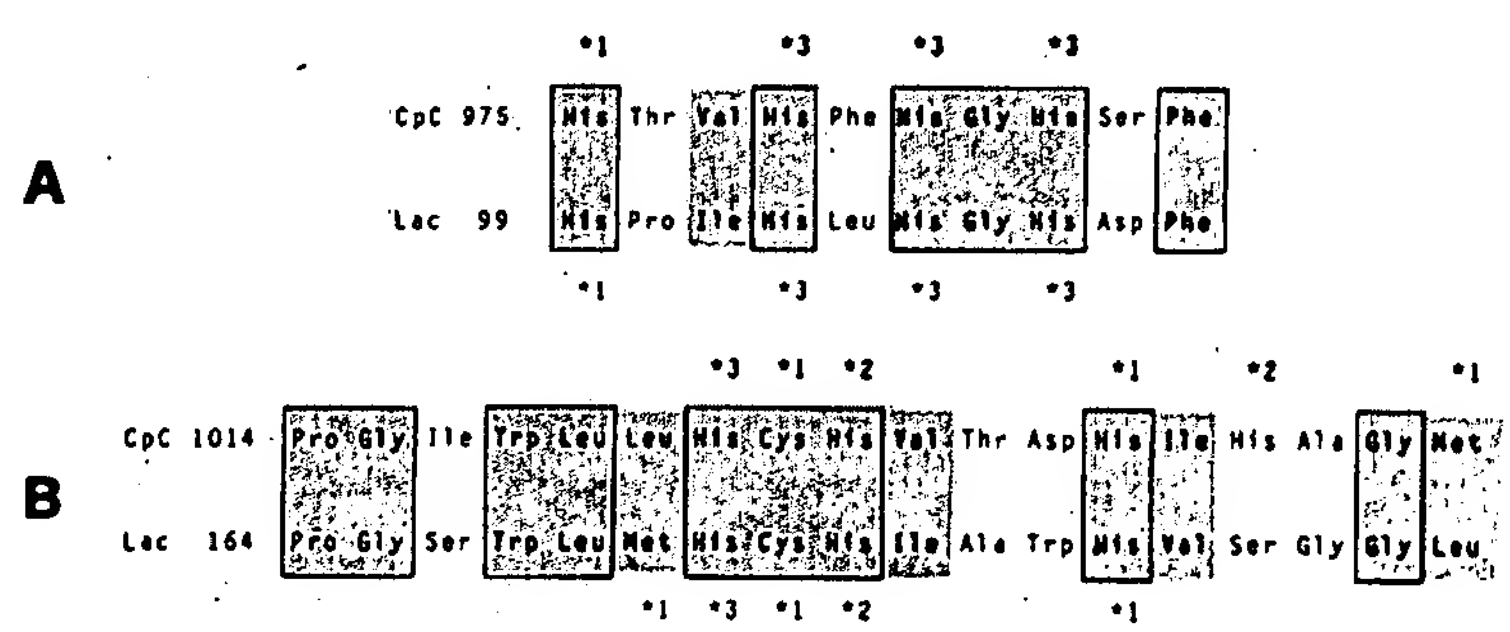

FIG. 6. Amino acid sequence comparison of two highly homologous regions (A and B) of *N. crassa* laccase (Lac) and the carboxyl-terminal homology unit (CpC) of human ceruloplasmin (46). Numbers on the left of each sequence identify the positions within the proteins of the first residues shown. For Lac, numbers refer to valine as 1, according to Fig. 4. Identical amino acid residues are boxed and isofunctional amino acid residues are shaded (see Fig. 5). Potential ligands to the three different types of copper are indicated by *1, *2, and *3, respectively. The assignment of the copper ligands for *N. crassa* laccase is based on that proposed earlier for CpC (47).

laccase that is homologous to the suggested type 2 copper binding His-1028 of ceruloplasmin. Hence, our data implicate that either the structures of the type 2 site in ceruloplasmin and laccase are different or the homologous type 2 ligands are closer to the amino terminus than suggested. It is clear that the complete amino acid sequence of *N. crassa* laccase and those of other multicopper oxidases are required to fully define the amino acid residues involved in the binding of the active site copper.

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